European Journal of Clinical Nutrition
https://doi.org/10.1038/s41430-020-0615-6

ARTICLE

Epidemiology

Circulating unmetabolized folic acid and 5-methyltetrahydrofolate and risk of breast cancer: a nested case-control study

Karen L. Koenig[1] · Stephanie Scarmo[1] · Yelena Afanasyeva[1] · Tess V. Clendenen[1] · Per Magne Ueland[2] · Anne Zeleniuch-Jacquotte[1,3]

Received: 9 December 2018 / Revised: 8 March 2020 / Accepted: 16 March 2020

Abstract

Background/Objectives Folates found in natural foods are thought to protect against cancer. However, folic acid (FA), a synthetic form of folate used in supplements and fortified foods, may increase breast cancer risk if present in unmetabolized form (UMFA) in the circulation. This study examined the associations of serum UMFA and 5-methyltetrahydrofolate (5-mTHF), the predominant form of circulating folate, with breast cancer risk.

Subjects/Methods We conducted a nested case-control study in a prospective cohort. In total, 553 cases of invasive breast cancer, diagnosed before mandatory FA fortification of grain in the US in 1998, were individually-matched to 1059 controls. Serum UMFA and 5-mTHF were measured using liquid chromatography-tandem mass spectrometry in stored serum samples, and 5-mTHF was corrected for storage degradation.

Results Serum UMFA was not associated with breast cancer risk: the percentage of women with detectable levels of UMFA was similar in cases and controls (18% and 20%, respectively; $p = 0.46$). Two tag-SNPs in the promoter region of the FA-metabolizing gene were also not associated with risk. There was a marginally significant inverse association of 5-$mTHF_{corrected}$ with breast cancer risk (odds ratio for the highest vs. lowest quintile = 0.69, 95% CI = 0.49 to 0.97; $p_{trend} = 0.08$).

Conclusions Circulating UMFA was not associated with breast cancer risk. These results apply to countries without mandatory FA food fortification. Studies are needed in countries with mandatory fortification, where levels of UMFA are much higher than in our study.

Introduction

Folate, one of the B vitamins, represents a family of chemically- and biologically-related compounds. Folate deficiency may lead to alterations in DNA synthesis, methylation, and repair, and could therefore play a role in carcinogenesis. Thus, it has been proposed that folate may protect against risk of cancer, including breast cancer [1], though some investigators have suggested a more complex relationship, with a protective effect against cancer initiation but promotion of already-initiated cancers [2]. A recent review by the World Cancer Research Fund Network concluded that the evidence regarding an association of folate intake with breast cancer risk was inconclusive [3].

Natural folates are found in a wide variety of foods, including vegetables (particularly dark green leafy vegetables) and fruits [4]. Most natural forms of folate are metabolized to 5-methyltetrahydrofolate (5-mTHF) during their absorption by the intestinal mucosa, and 5-mTHF is the main form of folate found in the circulation [5]. From the circulation, 5-mTHF enters cells where it is converted to THF as part of the methylation cycle; THF, in turn,

Supplementary information The online version of this article (https://doi.org/10.1038/s41430-020-0615-6) contains supplementary material, which is available to authorized users.

✉ Anne Zeleniuch-Jacquotte
anne.jacquotte@nyumc.org

[1] Departments of Population Health and Environmental Medicine, New York University School of Medicine, 650 First Avenue, New York, NY 10016, USA

[2] Department of Clinical Science, University of Bergen and Laboratory of Clinical Biochemistry, Haukeland University Hospital, 5021 Bergen, Norway

[3] Perlmutter Cancer Center, New York University School of Medicine, 530 First Avenue, New York, NY 10016, USA

Published online: 21 April 2020

participates in the de novo synthesis of thymidylate and purine, and thus in DNA synthesis [6].

Besides natural foods, supplements and fortified foods are also sources of folate. Until recently, folic acid (FA), a synthetic form of folate, was the only compound used for supplements, and it remains the only compound used in food fortification, because natural folates are unstable and rapidly lose their biological activity. FA requires two reduction reactions prior to becoming biologically active: the enzyme dihydrofolate reductase (DHFR) reduces FA first to dihydrofolate (DHF) and then to THF, primarily in the liver. THF is then methylated into 5-mTHF before entering the systemic circulation. It has been shown that the activity of DHFR can be overwhelmed/saturated at high intake of FA, resulting in the presence of unmetabolized FA (UMFA) in the circulation [7–9].

Results from two prospective cohort studies raised the question of whether high intake of FA may increase, rather than decrease, risk of breast cancer. In the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial, a statistically significant 19% increase in risk of breast cancer was observed among postmenopausal women reporting supplemental FA intake ≥400 µg/day [10]. In the Swedish Mammography Cohort, a statistically significant 19% increased risk of breast cancer was observed among multivitamin users, compared with nonusers (RR = 1.19; 95% CI = 1.04–1.37) [11]. The investigators concluded that FA could be responsible for the observed association, although they were unable to exclude a role from other nutrients found in multivitamins. Though other prospective studies found either no association between multivitamin use and breast cancer risk or a protective effect [12], other observations suggest that UMFA could increase cancer risk. High concentrations of UMFA in plasma have been found to decrease natural killer cell cytotoxicity, an immune response potentially promoting carcinogenesis [13, 14]. Furthermore, an increased risk of breast cancer was observed in women with a 19-bp deletion in the *DHFR* gene, which was reported to be associated with decreased functionality of the enzyme and higher levels of UMFA [15].

The U.S. Food and Drug Administration mandated FA fortification of grain products starting in 1998 to prevent neural tube birth defects [16]. As a result, intake of FA has substantially increased in the US population [17–19]. Folate supplementation is also mandatory or voluntary in a number of other countries. It is therefore important to examine the health effects of FA intake and circulating UMFA. To date, though, no epidemiologic study has assessed whether UMFA concentration in serum/plasma is associated with breast cancer risk.

The purpose of this study was to prospectively examine the association between the concentration of UMFA in serum and *DHFR* genetic variation with invasive breast cancer risk. We also examined the association of serum 5-mTHF, the predominant form of circulating folate, with invasive breast cancer risk.

Subjects and methods

Study population

A description of the New York University Women's Health Study has been provided previously [20]. Briefly, 14,274 healthy women were enrolled at a mammography screening clinic in New York City between 1985 and 1991. After giving written informed consent, all women completed a questionnaire that elicited information on medical and reproductive history, family history of breast cancer, and lifestyle factors, including diet and use of multivitamins. Blood samples were collected using standardized procedures, and serum was stored at −80 °C. Women who returned to the screening clinic during the enrollment period were asked to donate additional blood samples at each of these visits. The current study was approved by the Institutional Review Board of the New York University School of Medicine.

Case ascertainment and control selection

Incident cases of breast cancer are identified through active follow-up every few years using mailed questionnaires and telephone interviews for those who do not return the mailed questionnaire. Medical records are reviewed to confirm self-reported cases. Active follow-up is supplemented by linkage to state cancer registries in New York, New Jersey, and Florida (86% of the women resided in one of these 3 states at the latest follow-up) and the U.S. National Death Index. A capture-recapture analysis estimated a 95% case ascertainment rate in this cohort [21]. Invasive breast cancer cases diagnosed between enrollment and December 31, 1998 (the year when FA fortification of food supply became mandatory in the US) were eligible for this study ($n = 670$). We excluded cases diagnosed after mandatory fortification began because all of the NYUWHS blood samples were collected prior to fortification and would thus not be representative of the individual concentrations of UMFA and 5-mTHF after fortification, which could affect the participants' subsequent risk of breast cancer. Cases were excluded if they had any cancer prior to their breast cancer diagnosis ($n = 31$) or breast cancer diagnosed within 6 months after enrollment (prevalent cases, $n = 64$). An additional 22 cases were excluded due to low serum balance. As a result, a total of 553 cases were included in this study.

Two controls were selected for each case using incidence density sampling. Matching factors included age (±6 months) and menopausal status at enrollment, race/ethnicity, and date of enrollment/first blood donation (±3 months). Forty-seven of the selected controls had low serum balance and were therefore excluded. The total number of controls was therefore 1059.

Temporal reliability study

Prior to the case-control study, we conducted a pilot study to assess the temporal reliability of UMFA and 5-mTHF concentrations in our population. These analytes were measured in three yearly serum samples from 36 NYUWHS participants ($n = 108$ samples) who had no diagnosis of cancer or cardiovascular disease up to their latest date of follow-up. Per design, two-thirds of these women had reported using multivitamins at baseline.

Laboratory methods

UMFA and 5-mTHF serum concentrations were measured at the Bevital laboratory (Bergen, Norway, www.bevital.no) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [22]. Because of the long duration of storage (samples were stored for 21–27 years), possible degradation of 5-mTHF needed to be considered [23]. We therefore also measured 4-alpha-hydroxy-5-mTHF (hmTHF), which is produced upon degradation of 5-mTHF, and used the sum of the concentrations of hmTHF and 5-mTHF, denoted as 5-$mTHF_{corrected}$, as an estimate of the level of 5-mTHF in the circulation, corrected for storage-related degradation [24].

Samples from a case and her matched controls were analyzed in the same batch to minimize the impact of assay variability. Laboratory personnel were blinded to the case/control status of the samples. A total of 101 blinded quality control (QC) samples aliquoted from two pools were inserted randomly into the 21 batches. The two QC pools were created based on data from our temporal reliability study: one pool (31 QC samples) was created using serum samples with UMFA below the lower limit of detection (LOD, 0.3 nmol/L) of the assay, and the other (70 QC samples) using samples with UMFA levels above the LOD. All QC samples from the below-the-LOD pool were found to have levels below the LOD and all QC samples from the above-the-LOD pool were found to have levels above the LOD. For the 70 QC samples with detectable UMFA concentrations, the intra- and inter-batch coefficients of variation (CVs) were 4.4% and 6.4%, respectively. The intra- and inter-batch CVs were 1.8% and 2.3% for 5-mTHF, and 5.8% and 15.3% for hmTHF, respectively.

Genotyping *DHFR* variation

Based on sample type availability, DNA was extracted from clots, cell precipitates, or serum, using methods described in [25]. We had intended to examine the 19-bp deletion that had been reported to be associated with breast cancer risk in [15]. However, we failed to genotype this deletion using the method described in [8, 26] in a pilot study including samples from 158 NYUWHS participants (genotyping pilot set). Although we designed two different probe sets for RT-PCR analysis of this deletion and used gel-based genotyping methods, we could not distinguish between heterozygous and deletion genotypes. We therefore genotyped instead two tag-SNPs (rs844370 and rs11742668) in the promoter region of *DHFR* because tag-SNPs in the *DHFR* promoter region have been found to be associated with risk of colorectal cancer [27] and childhood acute lymphoblastic leukemia relapse [28]. We used TaqMan® assays [29, 30]. For these two SNPs, our pilot study (using the genotyping pilot set mentioned above) showed genotype calls >99% and concordance across DNA sample sources (serum, clots, or cell precipitates) >97%. Similar percentages of successful genotyping calls and duplicate concordance were observed in the case-control analysis, where blinded QC duplicates (9% of total samples) were interspersed throughout each plate. Samples from a matched case-control set were genotyped together on the same plate. DNA was unavailable from 42 cases and 161 controls, leaving 511 cases and 898 controls for the genetic analysis.

Statistical analysis

We used the yearly repeat measurements from our pilot study to assess the temporal reliability of the folate analytes. We used the intraclass correlation coefficient (ICC) for 5-mTHF and 5-$mTHF_{corrected}$. The ICC and corresponding 95% confidence interval (CI) were calculated using log-transformed values and a random-effects analysis of variance model with participant as a random variable. For UMFA, we calculated the weighted kappa statistic and percent agreement.

Because 81% of samples had serum UMFA levels below the assay LOD (0.3 nmol/L), UMFA was analyzed as a categorical variable (<LOD, ≥LOD and ≤ median of samples with detectable concentrations (2.01 nmol/L), or > median). Because 5-mTHF and 5-$mTHF_{corrected}$ were highly correlated (Pearson correlation coefficient = 0.99 for both cases and controls), we only report results for 5-$mTHF_{corrected}$. 5-$mTHF_{corrected}$ was log_2-transformed to reduce departure from the normal distribution. For the *DHFR* SNP analyses, CC or CT genotypes at rs11742668 were combined due to the small number of CC genotypes

(five cases and six controls), while an additive model was assumed for rs844370.

We used unconditional logistic regression to identify subject characteristics (including *DHFR* SNPs) associated with UMFA concentration (<LOD and ≥LOD), and linear regression to identify subject characteristics associated with 5-$mTHF_{corrected}$ concentration. These analyses were limited to control subjects. In addition to known breast cancer risk factors, we examined as possible predictors smoking, use of multivitamins, dietary folate intake, and hours since last meal. Dietary folate intake was estimated using the 70-item dietary questionnaire completed at blood donation, which included breakfast cereals, many of which were fortified with FA before 1998 [31, 32]. Variables that were statistically significantly associated with UMFA and 5-$mTHF_{corrected}$ in univariate analyses were entered in multivariate models to assess independent predictors of the concentrations of these analytes.

We used conditional logistic regression to estimate odds ratios (ORs) and 95% CIs for the association of the exposures of interest (UMFA in three categories, 5-$mTHF_{corrected}$ concentration in quintiles and continuous (log_2-transformed), and *DHFR* genotype) with risk of breast cancer. Likelihood ratio tests were used to assess statistical significance. All significance testing was two-sided, and p values < 0.05 were considered statistically significant.

We adjusted for the following breast cancer risk factors in multivariate models: age at menarche (continuous), age at first birth/parity (≤20, 21–25, 26–30, >30 years or nulliparous), history of breast cancer in a first-degree relative (mother, sister or daughter, yes/no), history of benign breast disease (yes/no), log_2-transformed body mass index (BMI), and alcohol consumption (0, <1, ≥1 drink/day). For continuous variables with a small number of missing values (age at menarche, BMI, and dietary folate), the median observed in controls was used for imputation, while a missing category was created for alcohol intake. There were no missing data for the other variables. An interaction term between BMI and menopausal status at baseline was included to take into account the different directions of the association of BMI with breast cancer risk in pre- and postmenopausal women [33].

We examined the association of $mTHF_{corrected}$ with risk according to estrogen receptor status of the tumor and time between blood donation and diagnosis. In addition, we conducted analyses stratified by the following baseline variables: age, menopausal status, education, BMI, alcohol consumption, and smoking. We also conducted analyses stratifying by multivitamin use, the main source of FA before mandatory food fortification, because the potential effect of *DHFR* polymorphisms is more likely to be observed among subjects exposed to high levels of FA. In order to avoid loss of data, analyses stratified by variables other than matching variables (age and menopausal status) were conducted using unconditional logistic regression and adjusting for the matching factors. Prior to conducting unconditional analyses, we verified that unconditional models with adjustment for the matching factors gave similar results to conditional models in analyses including all cases (Supplementary Table S1). To limit the problems associated with small numbers, subgroup and stratified analyses were conducted using continuous log_2(5-$mTHF_{corrected}$).

Results

Results from our temporal reliability study showed that a single measure of 5-mTHF (and 5-$mTHF_{corrected}$) was moderately representative of a subject's average concentration over a 2-year period (ICC = 0.57, 95% CI = 0.38–0.73 for 5-mTHF and ICC = 0.60, 95% CI = 0.42–0.75 for 5-$mTHF_{corrected}$). The weighted kappa statistic for UMFA in three categories (<LOD, ≥LOD to <median, ≥median) was 0.38 (95% CI = 0.09–0.66) and the coefficient of concordance was 0.62.

Descriptive statistics for breast cancer cases and matched controls are presented in Table 1. Subjects were between the ages of 35 and 65 years at enrollment, with a median age of 52 years. The median age at diagnosis was 60 years. For most known risk factors (younger age at menarche, nulliparity, older age at first full-term pregnancy, family history of breast cancer, history of benign breast disease, and, in postmenopausal women, overweight/obesity), the associations with breast cancer risk were in the expected direction, although not always statistically significant. Contrary to expectation, there was a marginally significant trend (p = 0.09) of lower alcohol consumption in cases than in controls. This was largely due to the difference in the proportions of non-drinkers (62% in cases vs. 56% in controls); only 13% of cases and 14% of controls reported at least one drink/day. The proportion of women who reported multivitamin use at enrollment was similar in cases (48%) and controls (50%). There was a suggestion (p_{trend} = 0.09) that fewer cases than controls had high dietary intake of folate.

Concentrations of the measured forms of folate in cases and controls are presented in Table 2. Only 19% of women had serum UMFA measures above the LOD, and the proportion was similar in cases and controls (18 and 20%, respectively; p = 0.46). The median concentrations of 5-$mTHF_{corrected}$ were slightly lower in cases than in controls (5-$mTHF_{corrected}$: 26.2 nmol/L, 10th–90th percentile: 13.1–66.7, and 27.8 nmol/L, 13.0–72.7, respectively; p = 0.02).

In multivariate analysis in control subjects (data not shown), determinants of UMFA above the LOD included multivitamin use, dietary folate intake, and older age (p <

Table 1 Characteristics of breast cancer cases ($n = 553$) and matched controls ($n = 1059$).

Characteristic	Case subjects *n* (%)	Control subjects *n* (%)	*p* value[a]
Age at enrollment, years			Matched
35–45	163 (29.5%)	331 (31.3%)	
46–54	147 (26.6%)	270 (25.5%)	
55–65	243 (43.9%)	458 (43.2%)	
Age at diagnosis, years			
≤55	212 (38.3%)		
56–64	163 (29.5%)		
≥65	178 (32.2%)		
Menopausal status at enrollment			Matched
Premenopausal	271 (49.0%)	525 (49.6%)	
Postmenopausal	282 (51.0%)	534 (50.4%)	
Race			Matched
Caucasian	427 (82.6%)	762 (79.2%)	
African-American	53 (10.2%)	102 (10.6%)	
Other	37 (7.2%)	98 (10.2%)	
Missing	36	97	
Education			0.29[b]
Some high school or less	18 (4.1%)	56 (6.5%)	
Completed high school	221 (50.9%)	445 (51.3%)	
College	104 (24.0%)	176 (20.3%)	
Graduate school	91 (21.0%)	190 (21.9%)	
Missing	119	192	
Age at menarche, years			0.22[b]
<12	122 (22.1%)	230 (21.8%)	
12	155 (28.1%)	269 (25.6%)	
13	167 (30.2%)	309 (29.3%)	
>13	108 (19.6%)	245 (23.3%)	
Missing	1	6	
Parous			0.08
Yes	367 (66.4%)	744 (70.2%)	
No	186 (33.6%)	315 (29.8%)	
Age at first full-term pregnancy, years			0.18[b]
≤20	50 (13.6%)	110 (14.8%)	
21–25	140 (38.1%)	337 (45.3%)	
26–30	107 (29.2%)	195 (26.2%)	
>30	70 (19.1%)	102 (13.7%)	
Ever used oral contraceptives			0.31
Yes	167 (35.2%)	349 (36.8%)	
No	307 (64.8%)	599 (63.2%)	
Missing	79	111	
Ever used hormone replacement therapy			0.71
Yes	107 (21.4%)	222 (22.2%)	
No	393 (78.6%)	778 (77.8%)	
Missing	53	59	
First-degree family history of breast cancer			0.18
Yes	133 (24.1%)	223 (21.1%)	
No	420 (75.9%)	836 (78.9%)	
History of benign breast disease			0.01
Yes	136 (25.0%)	198 (19.0%)	
No	417 (75%)	861 (81%)	
Body mass index (kg/m^2)			
Premenopausal			0.42[b]
<20.0	34 (12.6%)	59 (11.3%)	
20.0–24.9	147 (54.4%)	316 (60.3%)	
25.0–29.9	61 (22.6%)	104 (19.8%)	
≥30.0	28 (10.4%)	45 (8.6%)	
Missing	1	1	
Postmenopausal			0.01[b]
<20.0	12 (4.3%)	28 (5.3%)	
20.0–24.9	111 (39.6%)	271 (51.1%)	
25.0–29.9	118 (42.1%)	165 (31.1%)	
≥30.0	39 (13.9%)	66 (12.5%)	
Missing	2	4	
Ever smoker			0.83
Yes	256 (50.9%)	516 (52.6%)	
No	247 (49.1%)	465 (47.4%)	
Missing	50	78	
Alcohol, drinks/day			0.09[b]
0	287 (61.5%)	521 (55.7%)	
<1	120 (25.7%)	287 (30.7%)	
≥1	60 (12.8%)	127 (13.6%)	
Missing	86	124	
Multivitamin use			0.39
Yes	260 (48.1%)	520 (50.1%)	
No	281 (51.9%)	517 (49.9%)	
Missing	12	22	
Dietary folate (μg/day)			0.09[b]
<148.8	113 (21%)	211 (20%)	
148.8–204.3	113 (21%)	210 (20%)	
204.4–276.3	131 (24%)	210 (20%)	
276.4–365.1	93 (17%)	210 (20%)	
>365.1	94 (17%)	210 (20%)	
Missing	9	7	

[a]From conditional logistic regression.

[b]*p* for trend.

Table 2 Folate metabolite concentrations in breast cancer case and control subjects.

Biomarker	Case subjects ($n = 553$)	Control subjects ($n = 1059$)	p value[a]
UMFA			
n (%) above LOD	100 (18%)	207 (20%)	0.46
Median[b] (10th, 90th percentile)	1.86 (0.55, 15.4)	2.01 (0.62, 14.7)	0.39
5-mTHF, nmol/L Median (10th, 90th percentile)	21.9 (10.3, 62.3)	23.9 (10.5, 66.5)	0.02
hmTHF, nmol/L Median (10th, 90th percentile)	3.46 (1.39, 7.31)	3.37 (1.29, 7.58)	0.72
5-mTHF$_{corrected}$, nmol/L Median (10th, 90th percentile)	26.2 (13.1, 66.7)	27.8 (13.0, 72.7)	0.02

5-mTHF 5-methyltetrahydrofolate, *LOD* limit of detection, *UMFA* unmetabolized folic acid.

[a]From conditional logistic regression.

[b]For women with concentration ≥ LOD.

Table 3 Odds ratios and 95% CIs for breast cancer risk according to serum UMFA concentration (553 cases, 1059 controls).

	OR (95% CI)			p trend[a]
	<0.3 nmol/L (LOD)	0.3–2.01 nmol/L	>2.01 nmol/L	
Cases/Controls, n (%)	453 (82%)/852 (80%)	51 (9%)/103 (10%)	49 (9%)/104 (10%)	
Unadjusted model	1.00 (ref)	0.93 (0.65, 1.32)	0.88 (0.61, 1.27)	0.44
Adjusted model[b]	1.00 (ref)	1.04 (0.72, 1.49)	0.90 (0.62, 1.32)	0.69

[a]From conditional logistic regression.

[b]Adjusted for age at menarche (continuous), age at first full-term pregnancy/parity (ordered: ≤20, 21–25, 26–30, >30 years or nulliparous), family history of breast cancer (yes/no), benign breast disease (yes/no), BMI (log_2), BMI-menopausal status interaction, and alcohol consumption (0, <1, ≥1 drink/day, missing).

0.001 for these variables) as well as lower BMI and shorter time since last meal ($0.01 < p < 0.05$). The same variables were also predictors of circulating 5-mTHF$_{corrected}$ ($p <$ 0.001 for all variables).

Table 3 reports the ORs for breast cancer according to concentration of UMFA. We did not observe an association, either before or after adjustment for possible confounders. The adjusted odds ratio for women with concentration >2.01 nmol/L, as compared with levels below 0.3 nmol/L (LOD), was 0.90 (95% CI = 0.62–1.32; $p_{trend} = 0.69$).

The ORs for breast cancer by circulating levels of 5-mTHF$_{corrected}$ are presented in Table 4. A significant trend of decreasing risk with increasing concentration of 5-mTHF$_{corrected}$ was observed in unadjusted analysis ($p_{trend} = 0.03$), though the trend appeared driven largely by the top quintile (OR = 0.65, 95% CI = 0.47, 0.92). After adjustment for risk factors for breast cancer, the association was slightly attenuated and of borderline significance (OR for the highest vs. lowest quintile = 0.69, 95% CI = 0.49–0.97; $p_{trend} = 0.08$). When 5-mTHF$_{corrected}$ was analyzed on the continuous scale the adjusted odds ratio associated with a doubling in 5-mTHF$_{corrected}$ was 0.91 (95% CI = 0.81–1.02; $p_{trend} = 0.11$).

There was no evidence of heterogeneity in the 5-mTHF-breast cancer association when the data were stratified by estrogen receptor status of the tumor or time between blood collection and diagnosis (Table S2). There was also no evidence of heterogeneity by age, menopausal status at blood donation, education, alcohol consumption, multivitamin use or smoking status (Table S3). There was marginal evidence ($p = 0.06$) of heterogeneity by BMI in premenopausal women, with no association for women of normal weight but ORs below 1 for overweight and obese women. However, this relationship was not monotonic, with the OR associated with a doubling of 5-mTHF$_{corrected}$ of 0.55 (95% CI = 0.35–0.85) in women with BMI between 25 and 30 kg/m^2, and an OR of 0.78 (95% CI = 0.42–1.46) in women with BMI 30 kg/m^2 or higher.

For the two SNPs investigated, there was no evidence of departure from Hardy–Weinberg equilibrium, and the genotype frequencies were comparable to those observed in the 1000 genomes project (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). Table 5 shows ORs for breast cancer according to the *DHFR* SNPs. There was no association between either SNP and breast cancer risk (for rs844370: $OR_{GT\ vs\ TT} = 0.93$ (95% CI = 0.74–1.17), $OR_{GG\ vs\ TT} = 1.18$ (95%CI = 0.78–1.79), $p_{trend} = 0.87$; for rs11742668: $OR_{CT/CC\ vs\ TT} = 1.03$ (95%CI = 0.73–1.46), $p = 0.86$). Furthermore, there was also no association between these SNPs and breast cancer risk when the analysis was limited to either multivitamin users ($p_{trend} = 0.36$ for rs844370 and 0.67 for rs11742668) or nonusers

Table 4 Odds ratios and 95% CIs for breast cancer risk according to quintiles of 5-mTHF$_{corrected}$ and for a doubling in 5-mTHF$_{corrected}$.

	Quintiles[a]							Continuous (log_2(5-mTHF$_{corrected}$))
	1	2	3	4	5	p trend[b]	OR (95% CI)	p value[b]
Cases/controls	131/212	117/212	99/212	120/212	86/211		553/1059	
Unadjusted model	1.00 (ref)	0.88 (0.63, 1.23)	0.74 (0.53, 1.02)	0.90 (0.66, 1.24)	0.65 (0.47, 0.92)	0.03	0.89 (0.79, 0.99)	0.04
Adjusted model[c]	1.00 (ref)	0.86 (0.62, 1.21)	0.72 (0.51, 1.01)	0.91 (0.66, 1.25)	0.69 (0.49, 0.97)	0.08	0.91 (0.81, 1.02)	0.11

[a]Quintile cut points: 16.80, 23.85, 33.22, and 55.60 nmol/L.

[b]From conditional logistic regression.

[c]Adjusted for age at menarche (continuous), age at first full-term pregnancy/parity (ordered: ≤20, 21–25, 26–30, >30 years or nulliparous), family history of breast cancer (yes/no), benign breast disease (yes/no), BMI (log_2), BMI-menopausal status interaction, and alcohol consumption (0, <1, ≥1 drink/day, missing).

Table 5 Odds ratios and 95% CIs for breast cancer risk according to *DHFR* SNPs.

DHFR SNP	Cases	Controls	OR[a] (95% CI)	p trend
rs844370				0.87
TT	268 (53%)	460 (52%)	1.00 (ref)	
GT	195 (38%)	368 (41%)	0.93 (0.74, 1.17)	
GG	44 (9%)	63 (7%)	1.18 (0.78, 1.79)	
rs11742668				0.86
TT	451 (89%)	797 (89%)	1.00 (ref)	
CT/CC	58 (11%)	101 (11%)	1.03 (0.73, 1.46)	

[a]From conditional logistic regression, unadjusted model.

($p_{trend} = 0.47$ for rs844370 and 0.76 for rs11742668; data not shown). There was also no association between these SNPs and folate concentrations: neither the proportion of women with detectable UMFA in serum, nor the median concentration of 5-mTHF$_{corrected}$, varied significantly by genotype for either SNP (Table S4).

Discussion

We did not observe an association between serum UMFA and risk of breast cancer in this prospective study including cases diagnosed before mandatory FA fortification. We did observe a suggestive protective effect of 5-mTHF, the predominant circulating form of folate, on breast cancer risk. The two SNPs in the *DHFR* gene that we examined were not associated with concentrations of UMFA or 5-mTHF$_{corrected}$, or with risk of breast cancer.

Two prospective epidemiological studies have suggested that high FA doses may increase breast cancer risk [10, 11], and it has been hypothesized that this effect could be due to UMFA. Our results do not support this hypothesis, but are consistent with the results of a meta-analysis of 13 randomized trials including 50,000 individuals, which observed no association between FA supplementation and total or site-specific (including breast) cancer incidence during five years of treatment [34]. These results are also consistent with most observational studies that reported on intake of FA or use of multivitamins, the most common source of FA in many countries and in the US before fortification was implemented [12]. The lack of association between serum UMFA and breast cancer risk we observed is reassuring, particularly for countries that do not have mandatory fortification. Two limitations of our study need to be considered when interpreting these results. First, UMFA had a fairly low temporal reliability in our study: the kappa statistic was 0.38 (95% CI = 0.09–0.66), suggesting that a single blood measurement reflected imperfectly the average long-term concentration of UMFA in our population. This could have been due to changes over time in supplement intake in our participants. Another possible cause of the low temporal reliability is that blood samples were collected irrespective of the times since last meal and multivitamin intake, which are known to be inversely correlated with the concentrations of UMFA and other folate forms [35]. Low temporal reliability would be expected to lead to bias toward the null.

An additional limitation of our study is that the proportion of women with detectable UMFA was fairly small and thus our study had limited power to detect an association with breast cancer risk. This result is unlikely due to degradation of UMFA in storage because, though little is known regarding the effect of long-term storage, available data indicate that UMFA is very stable [36]. This result is also not due to low sensitivity of the assay because the LOD of the LC-MS/MS method we used was 0.30 nmol/L, similar to the LOD (0.28 nmol/L) of the LC-MS/MS method used in the 2011–2012 NHANES study that reported detectable UMFA in 99.9% of Americans aged ≥1 year [18]. All blood samples in our study were collected before 1998, and the low percentage of women with

detectable UMFA levels reflects the low FA intake in the US before mandatory food fortification. Though pre-post fortification changes in UMFA concentrations were not examined, NHANES data showed that serum folate concentrations nearly tripled after mandatory fortification in 1998 [37]. Studies using blood samples collected post-fortification are needed to assess the association of serum UMFA with breast cancer risk at levels representative of the current range of exposure in the US and other countries with mandatory FA food fortification.

Support for a detrimental role of UMFA in relation to breast cancer also came from the observation of an increased risk of breast cancer for women with a 19-bp deletion in the *DHFR* gene that was reported to be associated with decreased functionality of the enzyme and higher levels of UMFA [15]. Because we were not able to genotype this deletion, we examined two tag-SNPs in the promoter region of this gene. Neither SNP was associated with breast cancer risk. We also observed no association in analyses limited to women who used multivitamins, the main source of FA in our population. It is possible that FA intake was not high enough in our population to overwhelm DHFR activity, or that the genetic variants we captured are not associated with *DHFR* function, as their lack of association with concentration of UMFA suggests. The association of the *DHFR* 19 bp deletion with both UMFA concentration and breast cancer risk deserves further study. Though a recent large study concluded that this polymorphism does not significantly affect circulating folate status, contrary to [15], this study did not measure UMFA but only serum and red blood cell folate concentrations [38]. A comprehensive study of the factors, genetic and other, that affect UMFA concentrations would be of interest, in light of the fact that almost all individuals living in the US now have detectable levels of UMFA.

Five prospective studies previously evaluated the relationship between total folate concentration and breast cancer risk. Two studies [39, 40] reported a positive association in some subgroups, though these differed between the two studies. Two studies, including the large EPIC study, reported no association [41, 42]. One study reported a marginally significant reduced risk of breast cancer for women in the highest quintile of folate as compared with the lowest (RR = 0.73, 95% CI 0.50–1.07, p-trend = 0.06), an association which was highly significant for women consuming at least 15 g/d of alcohol [43]. Our results, which suggest an inverse association of breast cancer risk with 5-mTHF, are consistent with the results of this study, though we did not observe an interaction with alcohol consumption. Overall, studies to date are inconsistent. Furthermore, the folate concentrations in all six studies were much lower than those currently observed in the US population. This is not surprising since blood samples were collected before mandatory fortification in the four studies conducted in the US and the two other studies were conducted in Europe, where there is no mandatory fortification. While an extended duration of exposure at these higher concentrations may be required for an effect on breast cancer to be observed, prospective studies in the US using blood samples collected after 1998 should be able to detect an effect of high concentrations of folate, if such an effect exists, since 20 years have now elapsed since mandatory fortification was enacted. Studies in other countries using mandatory fortification should also be considered.

Strengths of this study include its prospective design and large sample size. Serum samples collected prospectively, prior to diagnosis, are necessary to assess the association of nutritional biomarkers in relation to subsequent breast cancer risk. In addition to serum samples, the NYUWHS also collected DNA, thus offering the opportunity to assess both circulating FA and tag-SNPs involved in its metabolism. To our knowledge, this is also the first study to examine serum concentrations of UMFA, in addition to 5-mTHF concentrations, in relation to breast cancer risk. Finally, we used LC-MS/MS which is the method of choice to assess individual folate vitamers [44, 45].

Results from this prospective study do not support the hypothesis that UMFA in the blood is associated with increased breast cancer risk. These results are reassuring for countries that do not have mandatory FA fortification of the food supply. However, much higher circulating concentrations of UMFA are observed currently in the US than we observed in our study. Furthermore, though the concentrations of folate vitamers other than UMFA have also increased substantially since 1998, no study to date has examined the impact of these changes on breast cancer risk. Because natural folates may have a different effect than FA, future studies should use methods, such as LC-MS/MS, that permit measurement of individual vitamers.

Funding This study was supported by research grants KG111502 from the Susan G. Komen Foundation, UM1 CA 182934 and P30 CA016087 from the US National Cancer Institute, and P30 ES000260 from the US National Institute of Environmental Health Sciences.

Author contributions KLK conceived of the study design, contributed to the analysis and interpretation of the data and to the writing of the manuscript. SS and AZJ conceived of the study design, acquired the data, contributed to the analysis and interpretation of the data and to the writing of the manuscript. YA analyzed the data and contributed to interpretation of the data. TVC selected the SNPs for analysis, assisted in the interpretation of the data and critically reviewed the manuscript. PMU performed the folate assays and critically reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Mason JB, Choi SW. Folate and carcinogenesis: developing a unifying hypothesis. Adv Enzym Regul. 2000;40:127–41.
2. Ulrich CM, Potter JD. Folate and cancer-timing is everything. JAMA. 2007;297:2408–9. https://doi.org/10.1001/jama.297.21.2408.
3. World Cancer Research Fund; American Institute for Cancer Research. Diet, nutrition, physical activity and breast cancer. Continuous Update Project Expert Report. 2018.
4. U.S. Department of Agriculture, Agriculture Research Service. USDA National Nutrient Database for Standard Reference, Release 25. 2012.
5. Shane B. Folate chemistry and metabolism. In: Bailey LB, ed. Folate in health and disease. New York: Marcel Dekker, 1995. pp 1-22.
6. Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF 3rd, Mills JL, et al. Biomarkers of nutrition for development—folate review. J Nutr. 2015;145:1636S–80S. https://doi.org/10.3945/jn.114.206599.
7. Wright AJ, Dainty JR, Finglas PM. Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr. 2007;98:667–75. S0007114507777140.
8. Kalmbach R, Choumenkovitch S, Troen A, Jacques P, D'Agostino R, Selhub J. A 19-base pair deletion polymorphism in dihydrofolate reductase is associated with increased unmetabolized folic acid in plasma and decreased red blood cell folate. J Nutr. 2008;138:2323–7.
9. Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr. 1997;65:1790–5.
10. Stolzenberg-Solomon RZ, Chang SC, Leitzmann MF, Johnson KA, Johnson C, Buys SS, et al. Folate intake, alcohol use, and postmenopausal breast cancer risk in the prostate, lung, colorectal, and ovarian cancer screening trial. Am J Clin Nutr. 2006;83:895–904.
11. Larsson S, Akesson A, Bergkvist L, Wolk A. Multivitamin use and breast cancer incidence in a prospective cohort of Swedish women. Am J Clin Nutr. 2010;91:1268–72.
12. Misotti AM, Gnagnarella P. Vitamin supplement consumption and breast cancer risk: a review. Ecancermedicalscience. 2013;7:365 https://doi.org/10.3332/ecancer.2013.365.
13. Troen A, Mitchell B, Sorensen B, Wener M, Johnston A, Wood B, et al. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr. 2006;136:189–94.
14. Sawaengsri H, Wang J, Reginaldo C, Steluti J, Wu D, Meydani SN, et al. High folic acid intake reduces natural killer cell cytotoxicity in aged mice. J Nutr Biochem. 2016;30:102–7. https://doi.org/10.1016/j.jnutbio.2015.12.006.
15. Xu X, Gammon M, Wetmur J, Rao M, Gaudet M, Teitelbaum S, et al. A functional 19-base pair deletion polymorphism of dihydrofolate reductase (DHFR) and risk of breast cancer in multivitamin users. Am J Clin Nutr. 2007;85:1098–102.
16. U.S. Food and Drug Administration. Food standards: amendment of standards of identity for enriched grain products to require addition of folic acid. Fed Register. 1996;61:44.
17. Pfeiffer CM, Sternberg MR, Fazili Z, Yetley EA, Lacher DA, Bailey RL, et al. Unmetabolized folic acid is detected in nearly all serum samples from US children, adolescents, and adults. J Nutr. 2015;145:520–31. https://doi.org/10.3945/jn.114.201210.
18. Pfeiffer CM, Sternberg MR, Fazili Z, Lacher DA, Zhang M, Johnson CL, et al. Folate status and concentrations of serum folate forms in the US population: National Health and Nutrition Examination Survey 2011-2. Br J Nutr. 2015;113:1965–77. https://doi.org/10.1017/S0007114515001142.
19. Pfeiffer CM, Johnson CL, Jain RB, Yetley EA, Picciano MF, Rader JI, et al. Trends in blood folate and vitamin B-12 concentrations in the United States, 1988 2004. Am J Clin Nutr. 2007;86:718–27.
20. Toniolo P, Levitz M, Zeleniuch-Jacquotte A, Banerjee S, Koenig KL, Shore RE, et al. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst. 1995;87:190–7.
21. Kato I, Toniolo P, Koenig KL, Kahn A, Schymura M, Zeleniuch-Jacquotte A. Comparison of active and cancer registry-based follow-up for breast cancer in a prospective cohort study. Am J Epidemiol. 1999;149:372–8.
22. Hannisdal R, Ueland PM, Svardal A. Liquid chromatography-tandem mass spectrometry analysis of folate and folate catabolites in human serum. Clin Chem. 2009;55:1147–54. https://doi.org/10.1373/clinchem.2008.114389.
23. Hannisdal R, Ueland PM, Eussen SJ, Svardal A, Hustad S. Analytical recovery of folate degradation products formed in human serum and plasma at room temperature. J Nutr. 2009;139:1415–8. https://doi.org/10.3945/jn.109.105635.
24. Hannisdal R, Gislefoss RE, Grimsrud TK, Hustad S, Morkrid L, Ueland PM. Analytical recovery of folate and its degradation products in human serum stored at -25 degrees C for up to 29 years. J Nutr. 2010;140:522–6. https://doi.org/10.3945/jn.109.116418.
25. Clendenen T, Zeleniuch-Jacquotte A, Wirgin I, Koenig KL, Afanasyeva Y, Lundin E, et al. Genetic variants in hormone-related genes and risk of breast cancer. PLoS ONE. 2013;8:e69367 https://doi.org/10.1371/journal.pone.0069367.
26. Johnson W, Stenroos E, Spychala J, Chatkupt S, Ming S, Buyske S. New 19 bp deletion polymorphism in intron-1 of dihydrofolate reductase (DHFR): a risk factor for spina bifida acting in mothers during pregnancy? Am J Med Genet A. 2004;124A:339–45.
27. Levine AJ, Figueiredo JC, Lee W, Conti DV, Kennedy K, Duggan DJ, et al. A candidate gene study of folate-associated one carbon metabolism genes and colorectal cancer risk. Cancer Epidemiol Biomark Prev. 2010;19:1812–21. https://doi.org/10.1158/1055-9965.EPI-10-0151.
28. Dulucq S, St-Onge G, Gagne V, Ansari M, Sinnett D, Labuda D, et al. DNA variants in the dihydrofolate reductase gene and outcome in childhood ALL. Blood. 2008;111:3692–700. https://doi.org/10.1182/blood-2007-09-110593.
29. Hui L, DelMonte T, Ranade K. Genotyping using the TaqMan assay. Curr Protoc Hum Genet. 2008;56(1):2.10.1–2.10.8.
30. Shen G, Luo A, Wang Q. High-throughput single-nucleotide polymorphisms genotyping: TaqMan assay and pyrosequencing assay. Methods Mol Med. 2006;128:209–24.
31. Kato I, Akhmedkhanov A, Koenig K, Toniolo P, Shore RE, Riboli E. Prospective study of diet and female colorectal cancer: The New York University Women's Health Study. Nutr Cancer. 1997;28:276–81.
32. Kato I, Dnistrian AM, Schwartz M, Toniolo P, Koenig K, Shore RE, et al. Serum folate, homocysteine and colorectal cancer risk in women: a nested case-control study. Br J Cancer. 1999;79:1917–21.
33. van den Brandt PA, Spiegelman D, Yaun SS, Adami HO, Beeson L, Folsom AR, et al. Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. Am J Epidemiol. 2000;152:514–27.
34. Vollset SE, Clarke R, Lewington S, Ebbing M, Halsey J, Lonn E, et al. Effects of folic acid supplementation on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data

on 50,000 individuals. Lancet. 2013;381:1029–36. https://doi.org/10.1016/S0140-6736(12)62001-7.
35. Haynes BM, Pfeiffer CM, Sternberg MR, Schleicher RL. Selected physiologic variables are weakly to moderately associated with 29 biomarkers of diet and nutrition, NHANES 2003–2006. J Nutr. 2013;143:1001S–10S. https://doi.org/10.3945/jn.112.172882.
36. Zayed A, Bustami R, Alabsi W, El-Elimat T. Development and validation of a rapid high-performance liquid chromatography-tandem mass spectrometric method for determination of folic acid in human plasma. Pharmaceuticals. 2018; 11. https://doi.org/10.3390/ph11020052.
37. Pfeiffer CM, Hughes JP, Lacher DA, Bailey RL, Berry RJ, Zhang M, et al. Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988–2010. J Nutr. 2012;142:886–93. https://doi.org/10.3945/jn.111.156919.
38. Osaki Y, Nakagawa Y, Miyahara S, Iwasaki H, Ishii A, Matsuzaka T, et al. Skeletal muscle-specific HMG-CoA reductase knockout mice exhibit rhabdomyolysis: a model for statin-induced myopathy. Biochem Biophys Res Commun. 2015;466:536–40. https://doi.org/10.1016/j.bbrc.2015.09.065.
39. Lin J, Lee IM, Cook NR, Selhub J, Manson JE, Buring JE, et al. Plasma folate, vitamin B-6, vitamin B-12, and risk of breast cancer in women. Am J Clin Nutr. 2008;87:734–43.
40. Ericson U, Borgquist S, Ivarsson MI, Sonestedt E, Gullberg B, Carlson J, et al. Plasma folate concentrations are positively associated with risk of estrogen receptor beta negative breast cancer in a Swedish nested case control study. J Nutr. 2010;140:1661–8. https://doi.org/10.3945/jn.110.124313.
41. Wu K, Helzlsouer KJ, Comstock GW, Hoffman SC, Nadeau MR, Selhub J. A prospective study on folate, B12, and pyridoxal 5'-phosphate (B6) and breast cancer. Cancer Epidemiol Biomark Prev. 1999;8:209–17.
42. Matejcic M, de Batlle J, Ricci C, Biessy C, Perrier F, Huybrechts I, et al. Biomarkers of folate and vitamin B12 and breast cancer risk: report from the EPIC cohort. Int J Cancer. 2017;140:1246–59. https://doi.org/10.1002/ijc.30536.
43. Zhang SM, Willett WC, Selhub J, Hunter DJ, Giovannucci EL, Holmes MD, et al. Plasma folate, vitamin B6, vitamin B12, homocysteine, and risk of breast cancer. J Natl Cancer Inst. 2003;95:373–80.
44. Fazili Z, Whitehead RD Jr., Paladugula N, Pfeiffer CM. A high-throughput LC-MS/MS method suitable for population biomonitoring measures five serum folate vitamers and one oxidation product. Anal Bioanal Chem. 2013;405:4549–60. https://doi.org/10.1007/s00216-013-6854-9.
45. Ueland PM, Midttun O, Windelberg A, Svardal A, Skalevik R, Hustad S. Quantitative profiling of folate and one-carbon metabolism in large-scale epidemiological studies by mass spectrometry. Clin Chem Lab Med. 2007;45:1737–45. https://doi.org/10.1515/CCLM.2007.339.